Full Length Research Paper

Kinetic growth of the isolated oleaginous yeast for microbial lipid production

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Microbial lipid was produced by the soil isolated oleaginous yeast *Torulaspora maleeae* Y30 using glucose in flask-batch fermentation. At low concentration of nitrogen, maximum lipid production rate was observed; while high nitrogen concentration (0.4 g/l) resulted in high volumetric cell mass production rate (0.960 g/l d) and specific growth rate (0.266 d^{-1}). The isolated yeast *T. maleeae* Y30 had maximum values of 0.382 g/l d, 0.507 g lipid/g cells, 3.06 g/l and 0.105 g/L d for volumetric lipid production rate, specific yield of lipid, lipid concentration and specific rate of lipid production, respectively when culture was performed in nitrogen-limiting medium supplemented with 90 g/l glucose and 0.2 g/l $(NH_4)_2SO_4$. The Monod's constant (K_S, g/l) and μ_{max} (1/d) of 24.52 and 0.156, were obtained, respectively. The three major constituent fatty acids of the isolated yeast *T. maleeae* Y30 were palmitic acid, stearic acid and oleic acid that are comparable to vegetable oils, suggesting that, microbial lipid from these yeast can be used as potential feedstock for biodiesel production.

Key words: Microbial lipid, oleaginous yeast, *Torulaspora maleeae* Y30, biodiesel.

INTRODUCTION

Biodiesel is defined as a mixture of mono-alkyl esters of long chain fatty acids derived from a renewable lipid feedstock, such as vegetable oil or animal fat. Biodiesel fuels are attracting increasing attention worldwide as a blending component or a direct replacement for diesel fuel in vehicle engines (Fukuda et al., 2001). Nowadays, there are increasing interests in looking for new oil feedstock for biodiesel production. Among them, microbial oils and lipid produced from oleaginous microorganisms, are now considered as promising feedstock because of their similar fatty acid composition to that of vegetable oils. The culture of these microbe species is affected neither by seasons nor by climates and can accumulate lipids within a short period of time as well as grow well on a variety of substrates (Hassan et al., 1996; Xue et al., 2006; Angerbauer et al., 2008; Li et al., 2008; Meng et al., 2009). Microbial oils, also referred to as single cell oils (SCO), are produced by many oleaginous micro-organisms involving yeasts, moulds and microalgae, which have ability to accumulate lipids over 20% of their biomass. Some oleaginous yeast strains, such as *Rhodosporidium* sp., *Rhodotorula* sp., *Lipomyces* sp. can accumulate intracellular lipids to level exceeding 70% of their biomass under nutrient limitation condition. The majority of those lipids are triacylglycerol (TAG) that contained long-chain fatty acids that

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Abbreviations: K_s, Monod's constant; **P,** lipid concentration (g/l); **Q_P,** volumetric lipid production rate (g/Ld); **Q_S,** volumetric substrate consumption rate (g substrate/L d); **Q_X,** volumetric cell mass production rate (g cells/L d); **q_p,** specific rate of lipid production (g lipid /g cells d); **q_s,** specific rate of substrate consumption (g substrate/g cells d); **S,** substrate concentration (g/l); **X,** cell mass concentration (g/L); **$Y_{P/S}$,** process product yield (g lipid/g substrate); **$Y_{P/X}$,** specific yield of lipid (g lipid/g cells); **$Y_{X/S}$,** cell yield coefficient (g cells/g substrate); **μ,** specific growth rate coefficient (1/d); **μ_{max},** maximum specific growth rate (1/d).

are comparable to conventional vegetable oils (Meng et al., 2009; Evan and Ratledge, 1984; Ratledge and Wynn 2002; Tehlivets et al, 2007). It is known that lipid production requires medium with an excess of sugars and limited other nutrients, usually nitrogen (Evan and Ratledge, 1984; Montet et al., 1985). Thus, oleaginous potential is critically affected by the carbon-to-nitrogen (C/N) ratio of the culture or lipid production is restricted when cultivation is carried out in nitrogen-limited sugar-based media (Evan and Ratledge, 1984; Montet et al., 1985; Turcotte and Kosaric, 1989; Hassan et al., 1996). It was also reported that the single cell oil production by oleaginous yeasts has many advantages due to their fast growth rate, high oil content and the resemblance of their TAG fraction to vegetable oil (Meng et al., 2009).

Kinetic models for microbial growth are classified in unstructured and structured models. The use of unstructured models is completely adequate in those cases where the substrate concentration is high compared with the saturation constant in the major part of batch fermentation. The Monod model is the most widely used and considered the basic equation of an unstructured model (Bailey and Ollis, 1986; Lee, 1992). This model introduced the concept of growth-limiting substrate (S), relating the specific growth rate (μ) to the concentration of a single growth-limiting substrate via two parameters, the maximum specific growth rate (μ_{max}) and the Monod's constant or saturation constant (K_s). The growth rate has been shown by Monod to be related to the concentration of substrate medium by the equation:

$$\mu = \frac{\mu_{max} S}{K_s + S} \qquad (1)$$

Where, μ is the specific growth rate; μ_{max} is the maximum specific growth rate unlimited by low concentrations of the substrate; S is substrate concentration; K_S is the concentration of substrate that supports a rate equal to $\mu_{max}/2$.

With the linearization method, the specific growth rate is determined by calculating the difference in the natural log of the biomass concentrations over time, corresponding to the exponential growth phase was plotted:

$$\mu = \frac{\ln(X_t - X_0)}{t} \qquad (2)$$

Where, X_0 is the biomass concentration at the beginning of the exponential growth phase; X is the biomass concentration at time t. Straight lines were obtained with slopes equal to μ and intercepts equal to lag phase time, for each set of experiments carried out.

The Lineweaver-Burk equation is obtained by taking the inverse of the Monod model:

$$\left(\frac{1}{\mu}\right) = \left(\frac{K_s}{\mu_{max}}\right) * \left(\frac{1}{S}\right) + \left(\frac{1}{\mu_{max}}\right) \qquad (3)$$

The slope is $\left(\dfrac{K_s}{\mu_{max}}\right)$ and the intercept is $\left(\dfrac{1}{\mu_{max}}\right)$ from

plotting $\left(\dfrac{1}{\mu}\right)$ versus $\left(\dfrac{1}{S}\right)$.

The Lineweaver-Burk method has been wildly used to determine the kinetic parameter values. However, transforming the variables often distorts the error associated with variables, because small errors for S (substrate) and the errors for $\left(\dfrac{1}{S}\right)$ become relative larger. Whenever $\left(\dfrac{1}{\mu}\right)$ versus $\left(\dfrac{1}{S}\right)$ are plotted, the error values may influence the slope significantly. This transformation is dependent on the value of the variable; therefore, it is not the most recommended method.

The Hanes plot is obtained by multiplying the Lineweaver-Burk equation by the substrate concentration.

$$\left(\frac{S}{\mu}\right) = \left(\frac{K_s}{\mu_{max}}\right) + \left(\frac{1}{\mu_{max}}\right) S \qquad (4)$$

The slope is $\left(\dfrac{1}{\mu_{max}}\right)$ and the intercept is $\left(\dfrac{K_s}{\mu_{max}}\right)$ of the

plot $\left(\dfrac{S}{\mu}\right)$ versus S.

The method is the most recommended for many different situations, because it minimizes the distortions in experimental error.

In microbial fermentation, variables which are of great relevance to the economic evaluation of biotechnological processes are the cell yield on a substrate ($Y_{X/S}$), specific growth rate (μ), volumetric substrate consumption rate (Q_S), specific substrate consumption rate (q_s), product yield based on substrate ($Y_{P/S}$), specific product yield ($Y_{P/X}$) and volumetric product formation rate (Q_P). All these kinetic parameters have major technological importance in up scaling the fermentation process (Tobajas and Garcia-Calvo, 1999).

In this study, the isolation of oleaginous yeasts from soil samples was reported using the glucose enrichment approach, then, growth kinetic parameters and lipid production of the isolated oleaginous yeast were investigated.

MATERIALS AND METHODS

Isolation and screening of oleaginous yeasts

The 40 soil samples were collected in the area of Chulabhorn Dam, Chaiyapoom province under Plant Genetic Conservation Project as The Royal Initiation of Her Royal Highness Princess Maha Chakri Sirindhorn (RSPG). A mass of 5.0 g of soil was added into 50 ml glucose-enriched medium containing (g/l): glucose (50), NH_4Cl (2.5), KH_2PO_4 (7.0), $MgSO_4.7H_2O$ (1.0), $CaCl_2$ (0.2), $FeCl_3.6H_2O$ (0.5 mg), $CuSO_4.5H_2O$ (0.5 mg), $ZnSO_4.7H_2O$ (0.7), yeast extract (2.0). The medium was supplemented with streptomycin 100 mg in a 250 ml flask and incubated in an incubator shaker at 30°C for 2 days with a shaking speed of 150 rpm. The strain isolation was investigated according to the serials dilutions technique and several inoculations were completed in YPG agar plates containing (g/l): glucose (20), peptone (10), yeast extract (10), agar (15) and the plates were then incubated at 30°C for 2 to 3 days. After pure cultures had been obtained, the isolated yeasts were initially cultivated onto YPG broth for 1 day at 30°C, then 5 ml of these culture were transferred to 250 ml flasks with 50 ml of nitrogen-limiting medium containing (g/l): glucose (70), $(NH_4)_2SO_4$ (0.1), KH_2PO_4 (0.4), $MgSO_4.7H_2O$ (1.5), $ZnSO_4$ (4.4 mg), $CaCl_2$ (25 mg), $MnCl_2$ (0.5 mg), $CuSO_4$ (0.3 mg) and yeast extract (0.75) and grown at 30°C in an incubator shaker at 150 rpm for 4 days. Duplicate samples were analyzed for cell dry weight, lipids and residual glucose. The isolated oleaginous yeasts with cellular lipid content more than 20% CDW were used for further study. Study of some characteristics of the isolated yeast was carried out according to the methods described according by the Yeast, A Taxonomic Study (Kurtzman and Fell, 1998).

Identification and genetic characterization

In order to identify the isolated yeast, sequence analysis of the variable D1/D2 domain of the large subunit (26S) ribosomal DNA was performed. The divergent D1/D2 domain of 26S rDNA was amplified with primers NL-1 (5'- GCA TAT CAA TAA GCG GAG GAA AAG-3') and NL4 (5'-GGT CCG TGT TTC AAG ACG G-3') (Kurtzman and Robnett, 1998). The polymerase chain reaction (PCR) reactions were performed in a final volume of 100 μl reaction mixtures conditioning 100 ng of genomic DNA, 2.5 U of Taq polymerase, 20 mM of each dNTP, 40 mM of each primer, 10 mM Tris-HCl and 1.5 mM $MgCl_2$. The reaction was pre-denatured at 94°C for 5 min, then repeated for 30 PCR cycles with denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 2.5 min and then followed by the final extension at 72°C for 10 min. The amplified product was then purified and sequenced. The 26S sequence of the isolated yeast was used for a BLAST search in the EMBL/GenBank database.

Seed culture preparation and culture conditions

The seed culture of the isolated oleaginous yeast was performed on YPG medium and grown at 30°C in an incubator shaker at 150 rpm for 2 days. Time course of cell growth was investigated on nitrogen-limiting medium supplemented with 70 g/l glucose. All the culture conditions were performed in 250 ml flask containing 50 ml of nitrogen-limiting medium and incubated in an incubator shaker at 30°C with 150 rpm.

To study the effect of initial pH medium, the seed culture (10%, v/v) was inoculated into nitrogen-limiting culture medium supplemented with 70 g/l glucose and pH value was adjusted from 4 to 6.

To study the effect of nitrogen concentration, the seed culture (10%, v/v) was inoculated into nitrogen-limiting medium supplemented with 70 g/l glucose and different concentrations of $(NH_4)_2SO_4$ were investigated.

To study the different glucose concentrations, the seed culture (10%, v/v) was inoculated into nitrogen-limiting medium supplemented with glucose to formulate a medium with an initial substrate concentration of 40, 50, 60, 70, 80, 90 and 100 g/l.

Analytical methods

The culture broth was centrifuged at 5,000 rpm for 5 min. The supernatants were analyzed for glucose concentration according to the DNS method (Miller, 1959). Harvested biomass was washed twice with distilled water and then dried at 90°C to constant weight. The biomass was determined gravimetrically. The lipid content was obtained by the ratio of total lipid concentration of biomass concentration. The total lipids were determined by the modified method of Know and Rhee (1986) with modifications. The fatty acid profile of the lipid was determined as fatty acid methyl esters (FAMEs) by the direct transesterification method with BF_3-methanol at 100°C for 45 min, reported by Lepage and Roy (1984). FAMEs samples were analyzed by gas chromatography (Shimadzu) equipped with a flame ionization detector (FID). The condition of GC analysis was as follows: 350°C FID, 40 ml/min N_2 carrier gas, 230°C injection port temperature, and 190°C oven temperature.

Determination of kinetic parameters

All kinetic parameters were determined as described earlier (Bailey and Ollis, 1986; Aiba et al., 1973). Volumetric product formation rate (Q_P) was determined from a plot between lipids (g/l) and fermentation time. The process product yield ($Y_{P/S}$) was determined from dP/dS and specific product yield ($Y_{P/X}$, g/g cell) was determined using the relationship dP/dX, while volumetric rate of substrate consumption (Q_S) was determined from a plot between substrate (g/l) present in the fermentation medium and time of fermentation. Volumetric cell mass production rate (Q_X, g dry cell/L d) was determined from a plot of dry cells (g/l) versus time of fermentation (d). The specific growth rate is the slope determined by plotting the natural log of biomass versus time for each substrate concentration during the initial phase of exponential growth before the substrate concentration decreases significantly, while specific rate of lipid production (q_P) was a multiple of μ and $Y_{P/X}$. Then, the determined values of the specific growth rate and substrate concentration determined are used to estimate the kinetics parameters, maximum specific growth rate (μ_{max}) and Monod's constant (K_s), with Hanes linear methods.

RESULTS AND DISCUSSION

The isolation and screening of oleaginous yeasts

In this preliminary study, 42 colonies with the morphology typical of yeast were isolated from 42 soil samples, then42 yeast colonies were tested for lipid accumulation on nitrogen-limiting medium supplemented with 70 g/l glucose and only one isolate namely Y30 was preliminary defined as an oleaginous yeast with lipid content of 22.7% of cellular dry weight (CDW). Therefore, the yeast

isolate Y30 was used for further study. The preliminary identification of yeast Y30 was ascomycetous yeast with diazonium blue B (DBB) and urease negative test.

Identification and genetic characterization

BLAST analysis of the 26S rRNA gene sequence of the yeast isolate Y30 was revealed to be a perfect match with that of *Torulaspora maleeae* type strain. The alignment and comparison of the 26S sequence of the isolate to the published 26S rRNA sequences belonging to five reference strains of phenotypically close species of *Torulaspora* confirmed the 99% correspondence to the *T. maleeae* CBS 10694, *T. maleeae* NBRC 103203, *T. maleeae* NBRC 103202, *T. maleeae* NBRC 103201 and *T. maleeae* NBRC 103200 type strains (Figure 1). In the 26S rDNA sequence result, as shown in Figure 2 the numbers of base substitutions between the yeast isolate Y30 and *T. maleeae* were zero (99% similarity).

The time course of cell growth, glucose utilization and lipid production of *T. maleeae* Y30 are shown in Figure 3. It is apparent that glucose was used mainly for cell growth at the beginning of the cultivation. Biomass, lipid content and utilized glucose gradually increased and lipid yield reached the maximum of 3.02 g/l or 47.19% CDW. A slight decrease was found in biomass after day 8, while the utilized glucose increased. The possible reason may be that nitrogen source was exhausted and a great deal of glucose consumption led to a decrease of pH, thus inhibiting cell growth. During the period between days 8 and 10, an apparent decrease in biomass and lipid content was observed. Similar changes were also observed in the lipid content *of Trichosporon fermentans*, after exhaustion of the carbon source in the growth environment (Zhu et al., 2008).

Effect of initial pH medium on cell growth and lipid production

Effect of initial pH on cell growth and lipid accumulation were investigated at the pH ranging from 4.0 to 6.0 (Figure 4). The maximum biomass (X) of 6.01 g/l with cellular lipid content of 46.67% CDW, were achieved at pH 5.0. For the experiments carried out in the culture medium with different initial pH, the kinetic parameters are shown in Table 1. The specific growth rate (μ) of each experiment was not significantly different. The maximum cell mass production rate (Q_X, g/l d), maximum lipid production rate (Q_P, g/l d) and specific yield of lipid ($Y_{P/X}$, g/g cell), were obtained as 0.751, 0.360 and 0.479, respectively, when the initial pH of the medium was adjusted to 5.0. However, at initial pH 5.0, the maximum substrate consumption rate (Q_S, g/l d) was found as 7.972, but maximum lipid yield coefficient ($Y_{P/S}$, g/g) or process product yield of 0.044 was observed.

Therefore, initial pH at 5.0 was selected for the next experiment.

Effects of nitrogen concentrations on cell growth and lipid production

The nitrogen concentration is one of the critical factors that have a profound influence on the lipid production in oleaginous microorganisms. The concentrations of 0.1, 0.2, 0.3 and 0.4 g/l $(NH_4)_2SO_4$ were used as the initial nitrogen source to investigate the effect on cell growth kinetic and lipid production. After 8 days of cultivation, higher initial nitrogen concentrations of the culture medium led to an increase in biomass concentration, with the maximum biomass concentration of 7.68 g/l obtained by the cultivation with an initial $(NH_4)_2SO_4$ of 0.4 g/l (Figure 5). In the experimental data, an increase in the $(NH_4)_2SO_4$ concentration of the culture medium led to a decrease in the lipid content of cells. The yeast *T. maleeae* Y30 had the highest total lipid content of 45.36% CDW by the cultivation with an initial $(NH_4)_2SO_4$ at 0.2 g/l.

The growth rate and lipid productivity of oleaginous yeast *T. maleeae* Y30 were strongly related to the nitrogen concentration. Kinetic and yield parameters of *T. maleeae* Y30 were calculated and the results are presented in Table 2. As can be seen, all parameter values were dependent on the nitrogen concentration. The increase in nitrogen concentration resulted in an increase in specific growth rate and $Y_{X/S}$ values and a decrease in lipid concentration (P). The maximum lipid production rate (Q_P, g/l d) was obtained at 0.373 when initial $(NH_4)_2SO_4$ concentration was 0.2 g/l with specific growth rate (1/d) of 0.236. Maximum biomass concentration (X, g/l) of 7.68 g/l was obtained at 0.4g/l of initial $(NH_4)_2SO_4$ concentration. The specific growth rate (μ, d^{-1}) obtained in these case was higher (0.266 d^{-1}) than that obtained for the initial nitrogen concentration of 0.1 (0.208 d^{-1}), 0.2 (0.236 d^{-1}) and 0.3 (0.255 d^{-1}) g/l, respectively. Volumetric cell mass production rate (Q_X, g/l d) of 0.779, 0.804, 0.885 and 0.960 were obtained using 0.1, 0.2, 0.3 and 0.4 g/l of nitrogen concentration. It was found that lipid production rate decreased as $(NH_4)_2SO_4$ concentration increased from 0.2 to 0.4 g/l. Consequently, initial concentration of $(NH_4)_2SO_4$ at 0.2 g/l, was considered to be appropriated to achieve high lipid production rate. The process product yields ($Y_{P/S}$, g lipid/g substrate) are equal to 0.043, 0.048, 0.041 and 0.037 in the culture medium with initial nitrogen concentration at 0.1, 0.2, 0.3 and 0.4 g/l, respectively. The growth rate and lipid accumulation of *T. maleeae* Y30 were strongly related to nitrogen concentration. Generally, the results confirmed that high nitrogen concentration supported maximum profiles of cell mass production rate and low nitrogen concentration supported maximum lipid production. Gradual reduction
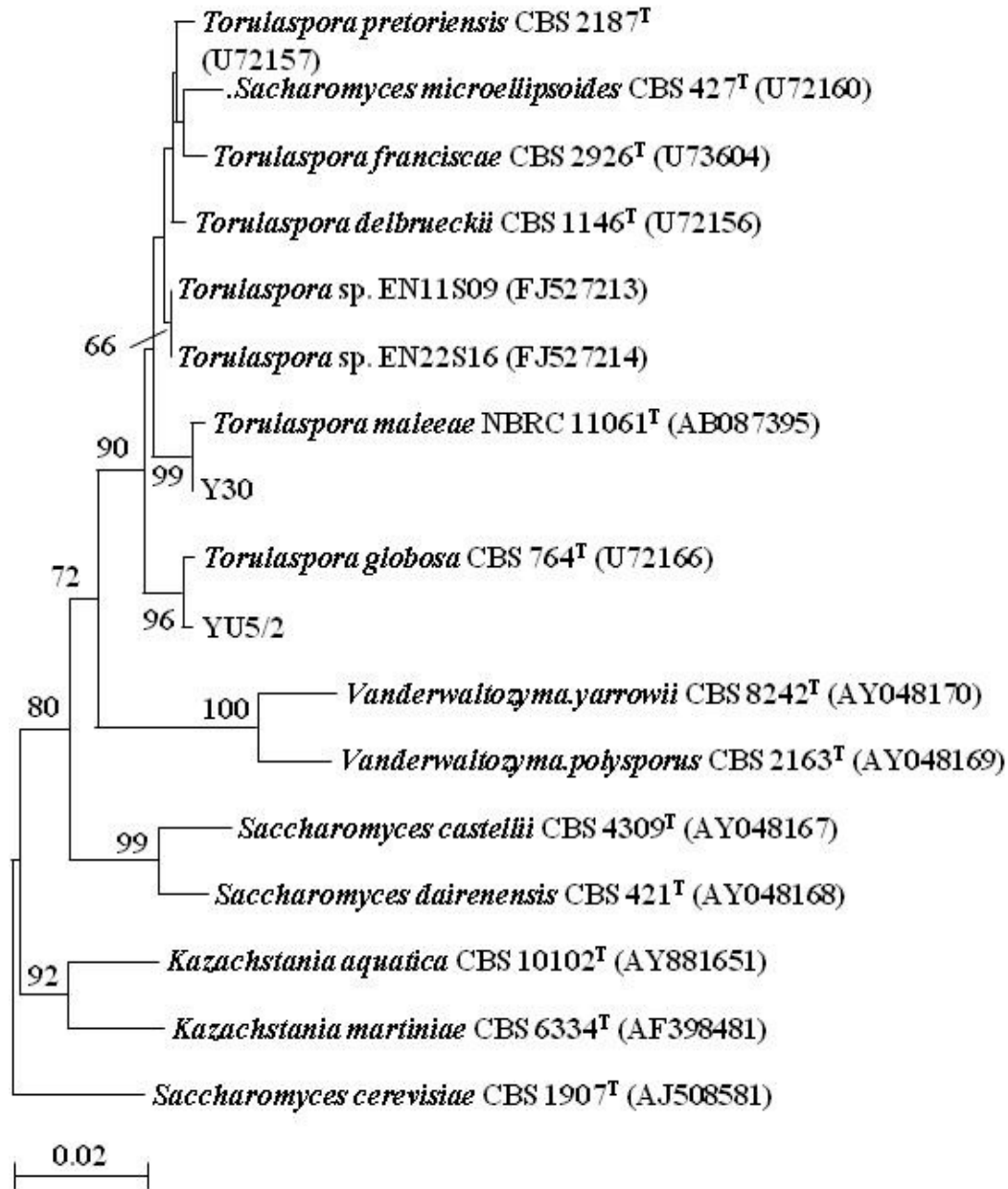
Figure 1. Phylogenetic tree based on the sequences of the D1/D2 domain of the LSU rRNA gene, showing position of the isolate Y30 with closely related species. The phylogenetic tree was constructed from the evolutionary distance data corrected by two-parameter transformation of Kimura (1980), using the neighbor-joining method. Numbers indicate percentages of bootstrap sampling, derived from 1000 samples.

in lipid formation, cell mass production and substrate consumption rates was observed when the nitrogen concentration was increased or decreased.

Effects of glucose concentrations on cell growth and lipid production

Batch flask cultures were investigated to determine the suitable glucose concentration of the initial medium.

Therefore, to study glucose concentrations on cell growth and lipid accumulation, the concentration of glucose at 40, 50, 60, 70, 80, 90 and 100 g/l with 0.2 g/l $(NH_4)_2SO_4$ were investigated. As shown in Figure 6 and Table 3, biomass decreased gradually with the increase of glucose concentration and the maximum biomass of 6.74 g/l with specific growth rate (μ) at 0.272 d^{-1} and cell yield coefficient ($Y_{X/S}$) at 0.200 was obtained at 40 g/l glucose, whereas low biomass of 5.91 g/l with specific growth rate at 0.171 d^{-1} was obtained at 100 g/l glucose. Cellular lipid

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Query  1    AAACCAACCGGGATTGCCTTAGTAACGGCGAGTGAAGCGGCAAAAGCTCAAATTTGAAAT  60
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Query  61   CTGGTACCTTCGGTGCCCGAGTTGTAATTTGTAGAAGGTAACTTTGGGGCTGGTCCTTGT  120
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Sbjct  541  GTCAAGGATGCTGGCATAATGGTTATATGCCGC  573
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Figure 2. Sequence alignment of the yeast isolate Y30 against D1/D2 of 26S rDNA sequence data of *T. maleeae* showing no base substitutions.
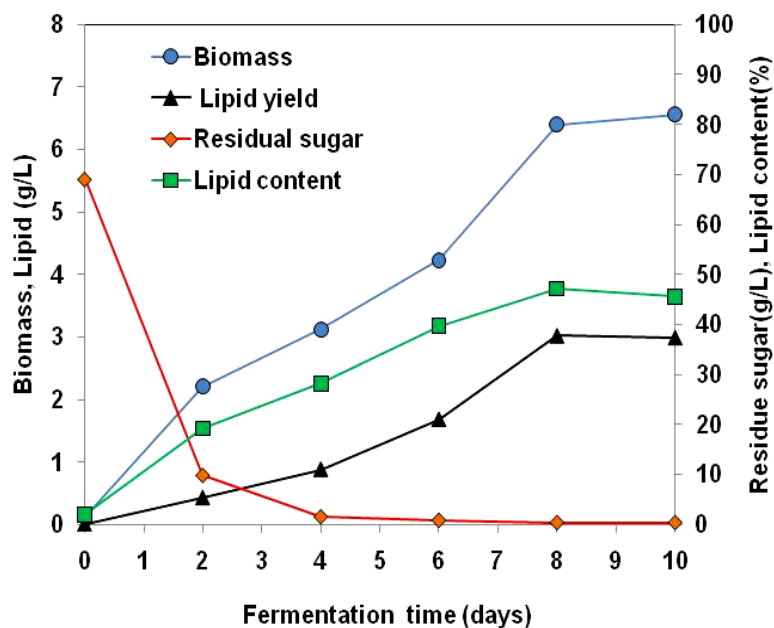

Figure 3. Time course of cell growth and lipid production in yeast isolate *T. maleeae* Y30. Culture was performed on nitrogen-limiting medium supplemented with 70 g/l glucose and 0.1 g/l $(NH_4)_2SO_4$.
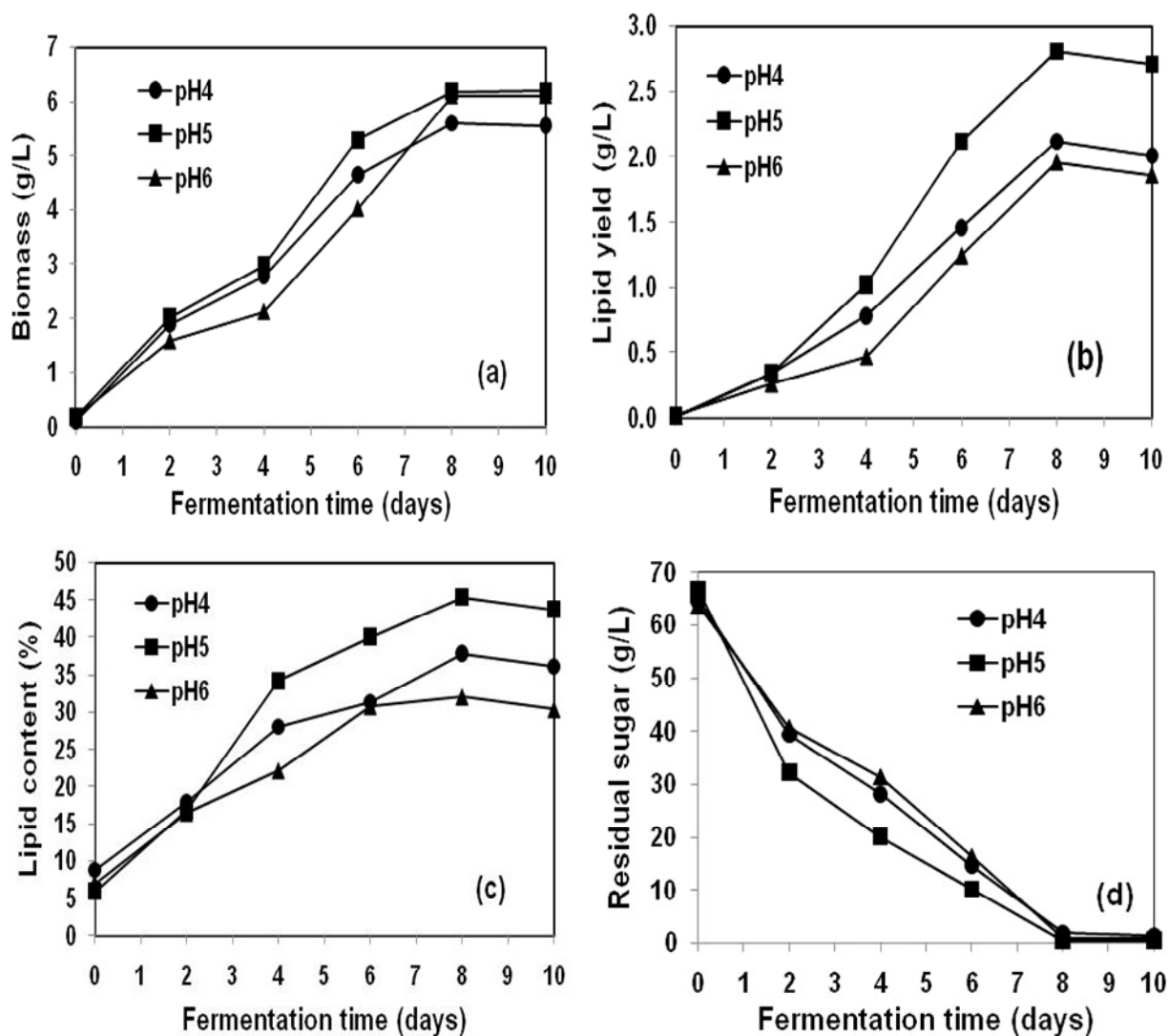
Figure 4. Effect of initial pH medium on biomass concentration. (a), lipid yield; (b), lipid content; (c) residual glucose; (d) fermentation of *T. maleeae* Y30 in nitrogen-limiting medium supplemented with 70 g/l glucose.

Table 1. Comparative fermentation kinetic parameters of *T. maleeae* Y30 grown on different initial pH of nitrogen-limiting medium (70 g/l glucose) in 250 ml flask at 30 °C.

pH	X (g/l)	P (g/l)	μ (1/d)	$Y_{X/S}$ (g/g)	$Y_{P/S}$ (g/g)	$Y_{P/X}$ (g/g cell)	Q_P (g/l d)	Q_X (g/l d)	Q_S (g/l d)
4	5.495	2.111	0.225	0.087	0.034	0.384	0.264	0.687	7.795
5	6.005	2.879	0.241	0.091	0.044	0.479	0.360	0.751	7.972
6	5.925	1.948	0.233	0.095	0.031	0.329	0.244	0.741	7.833

accumulation was quite low (1.98 g/l or lipid content of 23.44% CDW) at glucose concentration of 40 g/l, showed a sharp increase when glucose concentration increased from 50 to 90 g/l and reached the maximum of 3.02 g/l or 50.68% CDW at 90 g/l glucose with maximum specific yield of lipid ($Y_{P/X}$, g/g cells), specific rate of lipid production (q_P, g/l d) and lipid production rate (Q_P, g/l d)

of 0.507, 0.103 and 0.382, respectively. Further increase in glucose beyond 90 g/l resulted in a slight drop in lipid concentration and biomass, suggesting that a considerable glucose inhibitory effect had occurred. Indeed, glucose concentration has been found to be the major impact factor for oil accumulation by the oleaginous microorganisms (Papanikolaou et al., 2004). The
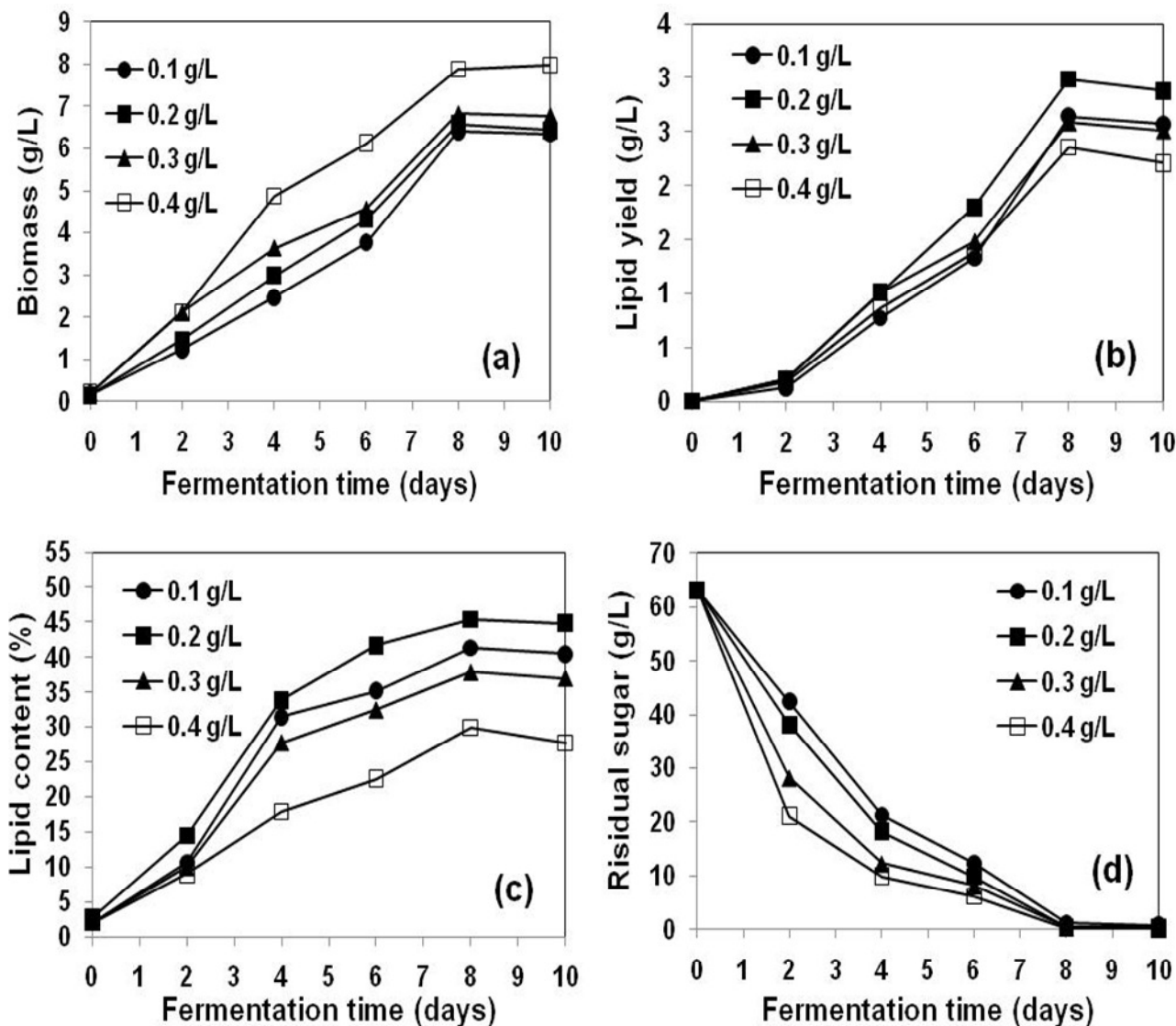
Figure 5. Effect of different nitrogen concentration on biomass concentration. (a), lipid yield; (b), lipid content; (c) residual sugar (glucose); (d) fermentation of *T. maleeae* Y30 in nitrogen-limiting medium supplemented with 70 g/l glucose.

Table 2. Comparative fermentation kinetic parameters of *T. maleeae* Y30 for lipid production on nitrogen-limiting medium supplemented with 70 g/l glucose (pH 5.0) in the presence of different nitrogen concentration in 250 ml flask at 30°C.

$(NH_4)_2SO_4$ (g/l)	X (g/l)	P (g/l)	μ (1/d)	$Y_{X/S}$ (g/g)	$Y_{P/X}$ (g/g)	$Y_{P/S}$ (g/g)	Q_P (g/l d)	Q_X (g/l d)	Q_S (g/l d)
0.1	6.23	2.861	0.208	0.101	0.424	0.043	0.358	0.779	7.742
0.2	6.43	2.981	0.236	0.103	0.464	0.048	0.373	0.804	7.830
0.3	7.08	2.579	0.255	0.113	0.364	0.041	0.322	0.885	7.834
0.4	7.68	2.354	0.266	0.122	0.294	0.037	0.306	0.960	7.860

determined values of specific growth rate and glucose concentration determined were used to estimate the kinetics parameters, maximum specific growth rate (μ_{max})

and Monod's constant or halt saturation constant (K_s), with Hanes linear methods. With fitted linear regression of Hanes plot (y = 6.3904x −157.16, R^2= 0.968), μ_{max} of
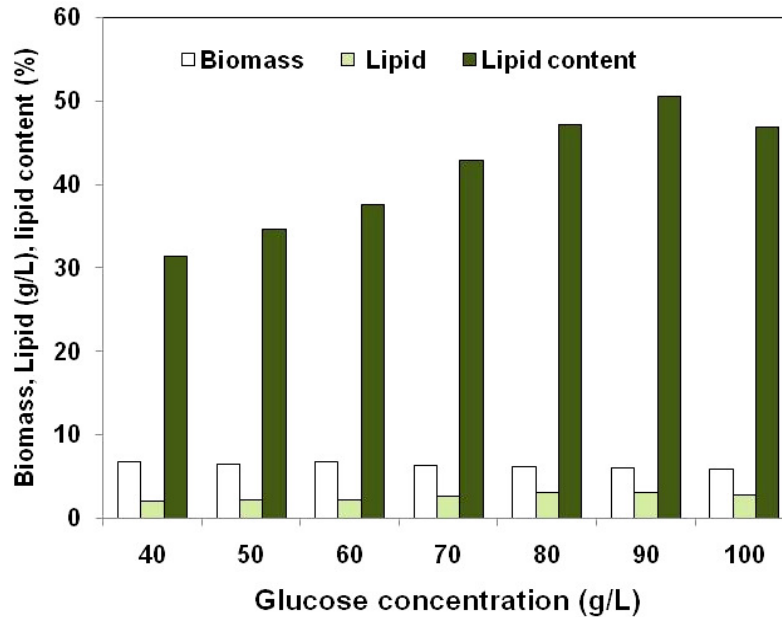
Figure 6. Effects of different concentrations of glucose on biomass, lipid yield and lipid content of *T. maleeae* Y30 in nitrogen-limiting medium at 30°C for 8 days in an incubator shaker with a shaking speed at 150 rpm.

Table 3. Comparative fermentation kinetic parameters of *T. maleeae* Y30 for lipid production on nitrogen-limiting medium supplemented with 0.2 g/l $(NH_4)_2SO_4$ (pH 5.0) in the presence of different glucose concentration in 250 ml flask at 30°C.

Kinetic parameter	Glucose concentration (g/l)						
	40	50	60	70	80	90	100
Biomass, X (g/l)	6.74	6.53	6.69	6.27	6.16	6.03	5.91
Lipid, P (g/l)	1.98	2.12	2.22	2.56	3.02	3.06	2.77
μ (1/d)	0.275	0.316	0.228	0.217	0.210	0.208	0.171
Q_X (g cell/L d)	0.842	0.816	0.836	0.784	0.770	0.753	0.739
$Y_{X/S}$ (g cell/g substrate)	0.200	0.148	0.125	0.103	0.083	0.078	0.072
$Y_{P/S}$ (g lipid/g substrate)	0.059	0.048	0.041	0.042	0.040	0.039	0.034
$Y_{P/X}$ (g lipid/ g cell)	0.294	0.325	0.332	0.408	0.489	0.507	0.468
$Q_{X/S}$ (g cell/g substrate d)	0.200	0.148	0.125	0.103	0.083	0.078	0.065
Q_P (g/l d)	0.248	0.265	0.278	0.320	0.377	0.382	0.346
Q_S (g/l d)	4.22	5.51	6.70	7.58	9.32	9.94	10.31
q_S (gS/g cell d)	0.626	0.843	1.001	1.209	1.512	1.608	1.744
q_P (g/l d)	0.081	0.103	0.076	0.088	0.103	0.105	0.080

0.156 (1/d) and K_s of 24.52 g/l were obtained.

However, the comparison of process product yield ($Y_{P/S}$) in the batch fermentation at high substrate concentration, showed that the increase of glucose concentration resulted in the decrease of this kinetic parameter and suggested that it was difficult for the up scaling of lipid production by the oleaginous organisms because high substrate consumption rate and high concentration of glucose with lower level of nitrogen source could affect the cell growth, because nitrogen source supported the cell growth, thus, depletion of nitrogen may result to low biomass. To solve these phenomena, further fed-batch fermentation should be investigated with initial nitrogen-rich medium to obtain high biomass or high cell density at the early stage of cell growth, and then high concentration of carbon source should be fed onto the culture medium to stimulate the cellular lipid accumulation. Fed-batch fermentation modes have been widely applied for microbial lipid production (Li et al., 2007).

When oleaginous organisms are grown with an excess of carbon and limited quantity of nitrogen, they may accumulate high concentration of cellular lipid. Carbon to nitrogen ratios (C/N ratio) in fermentation processes influence the formation of lipid concentrates. The growth of oleaginous yeast Y30 showed that biomass increase was followed by glucose concentration decrease and lipid production increased as nitrogen was limited. Cultivation of oleaginous yeast, with low nitrogen in the medium, results to the decrease of the activity of nicotinamide adenine dinucleotide isocitrate dehydrogenase (NAD-IDH) or even its disappearance from the mitochondria of the oleaginous yeasts; then the tricarboxylic acid cycle is repressed, metabolism pathway altered and protein synthesis stopped and lipid accumulation is activated (Evans et al., 1981; Botham and Ratledge, 1979; Palmieri et al., 1996).

Fatty acid profile analysis

GC analysis showed that the lipid extracted from *T. maleeae* Y30 mainly contained triacylglycerols (TAG) as 25.69% palmitic acid (C16:0), 23.39% stearic acid (C18:0), 45.41% oleic acid (C18:1), 3.41% linoleic acid (C18:2) and 1.83% linolenic acid (C18:3), which is similar to that of vegetable oils. The unsaturated fatty acids and saturated fatty acid amounted to about 49.31 and 50.69% of total fatty acid, respectively. Li et al. (2010) reported that the fatty acids from *Rhodotorula mucilaginosa* TJY15a were mainly composed of palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), oleic acid (C18:1) and linolenic acid (C18:2). Fatty acid of C16 and C18 and triglyceride (TG) dominated the crude lipid compounds of *Rhodotorula glutinis* cultivated on monosodium glutamate wastewater (Xue et al., 2008). Zhu et al. (2008) reported that the lipid of yeast *T. fermentans* mainly contains palmitic acid, stearic acid, oleic acid and linoleic acid and the unsaturated fatty acids amounted to about 64% of the total fatty acids.

Conclusion

The following conclusions can be drawn from this study; there seemed to be no lag phase during the growth of oleaginous yeast *T. maleeae* Y30 at either of the culture condition tested. Increasing nitrogen concentration resulted in a decrease in the lipid yield but increase in biomass yield. The experiment data obtained showed that cell growth and lipid production of yeast depended on the nitrogen and glucose concentrations; nitrogen supported cell growth while glucose played a role as the carbon source both for growth and lipid biosynthesis. The three major constituent fatty acids of *T. maleeae* Y30 were palmitic acid, stearic acid and oleic acid that are comparable to vegetable oils. Therefore, based on these fatty acid profile data, microbial oil from these yeast can be used as potential feedstock for biodiesel production. It has been known that, the costs of microbial oil production are currently higher than those of vegetable oil, but there are many methods to improve the low cost of microbial oil production processes. For example, the more economic carbon source should be employed to take the place of pure glucose as substrate such as sweet potato, new promoting plant oil, agro-industrial waste residues, that is, distillery slop (Zhao et al., 2010). In addition, potential and realistic progress in transforming lignocelluloses to fermentable carbon sources might provide an optimal way to reduce the cost of microbial oils production. Process engineering that leads to a higher lipid production rate and cellular lipid content may also contribute in this regard. Thus, to realize the large-scale production of biodiesel from microbial oils, it is necessary to obtain a large amount of biomass and lipid content via optimal fermentation process such as fed-batch fermentation as well as the low cost of cultivation process.

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